REMARKS

Claims 49-61 and 63-66 are pending and are rejected under 35 U.S.C. § 103. Claims 49, 51, and 58 are amended and new claims 68-72 are added.

Support for the Amendments

Support for new claims 67-72 is found in the specification and claims as originally filed. In particular, support for new claims 67 and 70, which recite GM-CSF, is found in Applicants' specification at page 4, line 32; support for new claims 68 and 69 is found at claim 1 as originally filed; support for new claim 69, which recites "an effective amount of a cytokine that mobilizes endothelial progenitor cells" is found, for example, at page 27, lines 27-27;" support for new claim 71, which recites "stem cell factor (SCF)" is found at page 5, line 1; and support for new claim 72, is found at page 20, lines 15-22; support for the amendment of claim 51, which now recites "VEGF-2" is found at page 21, lines 5-7.

Priority Claim

Applicants acknowledge the Examiner's objection to the priority claim. Applicants note that claims 49 and 58 no longer recite stem cell factor and are fully supported in Applicants' U.S. provisional application, which was filed on March 9, 1998, (U.S.S.N. 60/077,262, hereinafter "the '262 application"). Accordingly, the Office's objection to the priority claim with respect to claims 49-61 and 63-66 should be withdrawn.

In order to expedite prosecution and facilitate allowance, Applicants note that new claim 69 is directed to methods of treating myocardial ischemia by administering to the mammal an effective amount of granulocyte macrophage colony stimulating factor (GM-CSF) or an effective fragment thereof; and administering an effective amount of a nucleic acid encoding at least one angiogenic protein or an effective fragment thereof into the myocardial tissue; thereby treating the ischemic myocardial tissue of the mammal. Support for the administration of GM-CSF is found in the '262 application at page 4, lines 27 and 28, where Applicants state "These methods involve the use of GM-CSF to mobilize endothelial cell (EC) progenitors." Given that the '262 application clearly discloses methods of treating myocardial ischemia by injecting a nucleic acid encoding an angiogenic protein and administering to the mammal an effective amount of

granulocyte macrophage colony stimulating factor, the objection to the priority claim does not apply to claim 67.

In addition, Applicants have added new claim 69 to clarify that the invention is broadly directed to methods of treating myocardial ischemia by administering to a mammal an effective amount of a cytokine that mobilizes endothelial progenitor cells; and subsequently administering an effective amount of a nucleic acid encoding at least one angiogenic protein or an effective fragment thereof into the myocardial tissue; thereby treating the ischemic myocardial tissue of the mammal. The '262 application plainly provides support for new claim 68 at page 13, lines 13 and 14, where Applicants state "cytokine-induced EPC mobilization can enhance neovascularization" and at page 7, lines 18-21, where Applicants state:

> If it is desirable to further enhance angiogenesis, **angiogenic proteins, e.g., endothelial cell mitogens, may also be administered to the patient in conjunction with, or subsequent to, the administration of the GM-CSF**. The angiogenic protein can be administered directly, e.g., intra-arterially, intramuscularly, or intravenously, or nucleic acid encoding the mitogen may be used.

Accordingly, the Office's objection to the priority claim clearly does not apply to new claims 68 and 69.

Support for new claim 71 is found in the specification as originally filed. Accordingly, claim 71 is at least entitled to the filing date of the 09/265,041, which was filed on March 9, 1999.

Rejections under 35 U.S.C. § 103(a)

The Office rejects claims 49-61 and 63-66, which are directed to methods of treating an ischemic myocardial tissue by injecting GM-CSF or stem cell factor (SCF) and a nucleic acid encoding an angiogenic protein, under 35 U.S.C. § 103(a) as obvious over WO 97/14307 by Isner (hereinafter "Isner"), in view of U.S. Patent No. 5,880,090 by Hammond et al., (hereinafter "Hammond"); these claims are further rejected over Isner in view of Bussolino et al., (J. Clin. Invest. 87:986-995, 1991). Applicants respectfully disagree and for the reasons detailed below request that the obviousness rejection of the pending claims be withdrawn.

The test of obviousness requires that one compare the claimed "subject matter as a whole" with the prior art "to which said subject matter pertains" 35 U.S.C. § 103(a). To establish a *prima facie* case of obviousness, three criteria must be met. First, a suggestion or motivation to modify the reference or combine reference teachings must be present in the references or in the general knowledge present in the art. Second, there must be a reasonable expectation of success. Finally, the prior art reference must teach or suggest all the claim limitations. M.P.E.P. 2143. The burden is on the Office to show that the references expressly or impliedly suggest all of the claim limitations. M.P.E.P. 2142. "There are three possible sources for a motivation to combine references: the nature of the problem to be solved, the teachings of the prior art, and the knowledge of persons skilled in the art." *In re Rouffet*, 149 F.3d 1350, 1357. In the absence of some teaching or suggestion to combine, no *prima facie* case of obviousness can be established, and the rejection is improper and must be withdrawn. *In re Fine*, 837 F.2d 1071, 1074.

In the present case, the references cited by the Office fail to provide the requisite motivation to combine; fail to provide a reasonable expectation of success; and fail to teach or suggest all of the claim limitations. Each of the references cited by the Office in support of the obviousness rejection is considered below.

Isner

Isner describes methods for treating ischemic tissue. The Office states:

> ***Isner does not specifically teach the administration of an effective amount of a stem cell factor, a colony stimulating factor or an effective fragment thereof*** into the mammal with an effective amount of a solution comprising a nucleic acid encoding at least one angiogenic protein or an effective fragment thereof. (Office action mailed January 25, 2006; page 4, last paragraph)

The Office indicates that Isner fails to disclose a method of treating ischemic myocardial tissue with a colony stimulating factor as recited in claims 49-61 and 68. To the extent that such subject matter is not taught or suggested by Isner, the Office relies on Hammond to remedy the alleged failure of Isner.

Hammond

Hammond describes methods for coating a ***synthetic vascular graft*** with endothelial cells. Hammond fails entirely to teach or suggest methods for treating myocardial ischemia as recited by Applicants' claims. Regarding Hammond, the Office asserts that i) the methods of Hammond are indistinguishable from those described by Applicants; ii) Hammond teaches methods for enhancing blood vessel formation in a patient; and iii) one of skill in the art would be motivated to combine the methods taught by Hammond with the methods described by Isner. Applicants respectfully disagree with these assertions.

I. Methods for enhancing endothelialization of a synthetic graft are readily distinguishable from methods of treating an ischemic tissue

The methods described by Hammond are uniformly directed to methods for coating ***synthetic grafts*** with endothelial cells; such methods are readily distinguishable from Applicants' claimed methods, which are directed to methods for treating ischemic myocardial ***tissue of a mammal***. The synthetic grafts described by Hammond comprise polyethylene terephthalate and polytetrafluoroethylene. The usefulness of such grafts is limited by their tendency to induce clot formation (column 1, lines 15-26). To overcome such limitations, Hammond describes methods for increasing the number of endothelial cells that ***attach to and coat the surface*** of synthetic grafts. Hammond states, "circulating cells that give rise to ***endothelial coatings of vascular prostheses*** may arise from the bone marrow. (column 1, line 60, to column 2, line 5; emphasis added; citations deleted.) Methods for increasing the number of endothelial cells that attach to a graft to form an endothelial coating are distinctly different from Applicants' claimed methods, which provide for the treatment of myocardial ischemia in a ***patient***. The formation of ***endothelial coatings on synthetic grafts*** described by Hammond can in no way make obvious Applicants' claimed methods.

II. Hammond fails to teach methods for enhancing endothelialization in a tissue of a patient.

The Office asserts that Hammond teaches methods for enhancing endothelialization of tissues in a patient. Specifically, the Office states that Hammond showed that "mobilization of endothelial cell progenitors would further enhancing blood vessel formation or angiogenesis in an ischemic tissue." (Office action mailed January 25, 2006, page 8, lines 11-14.) Applicants

respectfully disagree. In fact, Hammond fails to describe any method of enhancing endothelialization in a ***tissue or organ*** of a patient, as Applicants do. As detailed above, the methods described by Hammond merely provide for ***endothelial coatings of synthetic materials*** to reduce thrombus formation. (column 2, lines 53-67).

Not only are such synthetic grafts plainly distinct from the tissues of a mammal, but the formation of endothelial coatings is also distinct from the formation of blood vessels within a tissue. Hammond teaches that endothelialization promoting agents (e.g., GM-CSF, G-CSF) enhance "**adherence of circulating endothelial cells to graft surfaces,** or may stimulate the multiplication of blood-borne endothelial precursors that have become **adhered.**" (column 2, lines 64-67.) Hammond teaches that this process relies on "'fallout endothelialization.' More specifically, it has been proposed that the circulating cells that give rise to endothelial coatings of vascular prostheses . . . " Methods for coating a synthetic prostheses with a layer of endothelial cells are readily distinguishable from the multifaceted biological process that regulates blood vessel formation within a tissue or organ.

Applicants' specification teaches that the formation of blood vessels in a tissue involves the complex regulation of a variety of endothelial cell functions and activities, including cell migration, proliferation, the formation of endothelial cell sprouts, vascular loop formation, the development of capillary tubes and the subsequent formation of tight junctions and the deposition of new basement membranes (page 2, lines 10-15). Such vascular networks fulfill a critical biological function within the tissue of the subject by providing oxygen and nutrients and removing wastes (page 1, lines 27-30). Hammond's process of coating a synthetic graft with endothelial cells is plainly different from the process of blood vessel formation.

To expedite prosecution and clarify the distinguishing features of the present invention, Applicants note that new claim 68 recites that the method increases the neovascularization of the tissue. Accordingly, the obviousness rejection over Isner in view of Hammond should not apply to claim 68.

III. Hammond teaches away from combining the methods described by Isner with methods for promoting endothelialization.

The endothelialization results obtained by Hammond fail to provide the requisite motivation to combine or the expectation of success to modify the methods of Isner. A thorough reading of Hammond suggests that methods for promoting endothelialization may have undesired side effects that would dissuade the skilled artisan from utilizing the methods described by Hammond. In particular, Applicants invite the Office's attention to Example 1, where Hammond describes grafts having endothelial coatings. Regarding such grafts, Hammond states,

> [T]he BMB grafts implanted for four weeks or longer appeared stiff. Histological studies revealed many **osteocytes with microcalcification in the outer graft wall** of these grafts, but not in the inner wall or intima, even at three months. **In the BMB grafts implanted longer than four weeks, osteoblasts, osteocytes, and microcalcifications were found. These undesirable side effects could affect the long-term utility of such grafts . . .** (column 7, lines 55-63)

Hammond's disclosure of adverse results associated with the endothelialization of grafts teaches away from the use of such methods. In view of this teaching away, one skilled in the art would lack the requisite motivation to introduce changes to the methods of Isner, and would further lack the expectation of success required to introduce such changes.

In the Advisory action mailed July 10, 2006, the Examiner asserts that *in vivo* studies failed to show these undesirable side-effects. Applicants respectfully disagree. Hammond merely states that two dogs that received synthetic grafts had 80% and 35% of their graft surfaces covered with endothelial like cells (Column 9, lines 48-50). This summary in Example 3 fails to indicate whether or not the grafts were evaluated for the presence of microcalcification. Furthermore, Example 4 fails to provide any description of results.

In sum, Hammond fails to teach or suggest any method for **treating myocardial ischemia in a tissue** of a subject, much less Applicants' claimed methods, which recite identifying a patient in need of such treatment, injecting a nucleic acid molecule encoding an angiogenic factor, and administering GM-CSF or SCF (claim 71). Moreover, one skilled in the

art would lack the requisite expectation of success to combine the methods of increasing synthetic graft endothelialization described by Hammond with any other method described in the references cited by the Office.

Bussolino

Bussolino describes the use of G-CSF and GM-CSF to induce proliferation and migration in endothelial cells. The Office asserts that it would be obvious to modify the methods of Isner by utilizing recombinant G-CSF as described by Bussolino. In support of the rejection, the Office states:

> An ordinary skilled artisan would have been motivated to carry out the above modification because Bussolino et al. already demonstrated that recombinant G-CSF has angiogenic activity *in vivo*, and that it also exhibits synergistic effects with at least another endothelial cell mitogen bFGF in inducing in vivo angiogenesis.

Applicants respectfully disagree. Bussolino fails to teach or suggest any method of treating **myocardial ischemia**, much less Applicants' claimed methods which require identifying a mammal which has or is suspected of having an ischemic tissue; injecting an effective amount of a solution comprising a nucleic acid encoding at least one angiogenic protein or an effective fragment thereof into the myocardial tissue; and administering to the mammal an effective amount of a colony stimulating factor thereby treating the myocardial ischemia.

I. Bussolino shows that G-CSF has only a weak angiogenic effect *in vivo*, and fails to conclude that G-CSF and bFGF act cooperatively

Contrary to the Office's assertion, Bussolino shows that when G-CSF is administered alone it exhibits only **weak angiogenic** activity *in vivo*. At page 994, right column, lines 2 and 3, Bussolino states, "G-CSF had ***relatively weak***, but definite, angiogenic activity in the rabbit cornea:" (emphasis added.) The weak angiogenic activity described by Bussolino would be insufficient to motivate the skilled artisan to adapt the methods taught by Isner by including G-CSF, particularly where Bussolino states that G-CSF was less active than bFGF (page 986, Abstract). Moreover, Bussolino fails to conclude that G-CSF and bFGF exhibit a synergistic effect. Rather, Bussolino states that the observed effects are ***merely suggestive*** of a cooperative

effect. At page 994, lines 12-15, Bussolino states, "By combining nonangiogenic doses of bFGF with G-CSF, we observed responses whose intensity is ***suggestive*** of a cooperative interaction of the two cytokine in inducing angiogenesis (emphasis added). Thus, Bussolino fails to conclusively determine the nature of the interaction between bFGF and G-CSF.

II. Bussolino emphasizes the preliminary nature of the G-CSF and bFGF results

In addition, Bussolino fails to teach or suggest that G-CSF should be used to enhance angiogenesis in other tissues. In fact, Bussolino indicates that further studies are required to characterize the effects of G-CSF on angiogenesis. Rather than concluding that G-CSF should be used to enhance angiogenesis in any tissue, Bussolino repeatedly emphasizes the preliminary nature of the G-CSF and bFGF results. Bussolino states, "we wanted to obtain ***initial indications*** as to the capacity of this cytokine to act in concert with bFGF." Bussolino also states that "This ***initial observation*** needs to be ***extended***." Clearly, Bussolino indicates that his results are not the endpoint of exhaustive studies, but are merely preliminary indications, which provide a jumping off point for further study.

III. Bussolino teaches that it is difficult to predict effects on *in vivo* angiogenesis

Nor does Bussolino indicate that these studies are broadly applicable to methods for enhancing angiogenesis in a variety of tissues *in vivo*, particularly **myocardial tissue** as recited in Applicants' claims. In fact, Bussolino stresses that it is difficult to predict effects on *in vivo* angiogenesis given the complexity of the biological processes involved. Bussolino states:

> *In vivo* angiogenesis occurs as the end point of ***complex interactions*** between many events involving the remodeling of the extracellular matrix and the release of several "factors". ***This apparent paradox of a combination of cytokines acting directly on endothelial cells, showing a cooperative effect in vivo, but not in vitro***, adds to the list of factors or conditions for which ***in vitro modulation of proliferation and migration is not necessarily predictive of in vivo effects on angiogenesis***. Possible explanations for this partial ***discrepancy in the capacity of G-CSF to act in concert with bFGF in vitro and in vivo*** could involve a different biology of microvascular endothelium versus HUVEC or ***effects of G-CSF on passing neutrophils***.

Bussolino indicates that his observations concerning angiogenesis are influenced by complex and unpredictable interactions that are liable to be ***influenced even by the effects of passing neutrophils***. Given the paradoxes and uncertainties that exist in the results of Bussolino,

Bussolino fails to provide the motivation or expectation of success required to adapt the methods of Isner. In particular, Bussolino fails to teach or suggest that such methods should be used for treating **myocardial ischemia**.

The standard in determining obviousness is not whether certain experiments ***could be tried***, but whether the prior art suggested that the modifications ***should be made***, and further suggested that the modified methods ***would function successfully***. *In re Dow Chemical Co.*, 837 F.2d 469 (Fed. Cir.,1988). In this case, the court held:

> The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that ***this process should be carried out*** and would have ***a reasonable likelihood of success***, viewed in the light of the prior art. Both the suggestion and the expectation of success must be founded in the prior art not in the applicant's disclosure. *Id* at 473. (citations omitted; emphasis added.)

None of the references cited by the Office teaches or suggests each of the elements recited in Applicants' claimed invention. None teaches that one should administer GM-CSF or SCF in combination with an angiogenic factor for the treatment of an ischemic myocardial tissue. It is not sufficient that one ***could*** have made the combination, the cited references must suggest the desirability of making the claimed combination and must further indicate that the combination if made would have succeeded.

In sum, Applicants were the first to appreciate that myocardial ischemia could be treated by injecting a myocardial tissue with a nucleic acid encoding an angiogenic factor and administering GM-CSF or SCF. None of the references cited by the Office, alone or in any combination, teaches or suggests all of the claimed limitations of Applicants' claimed invention. The Office has failed to establish a *prima facie* case of obviousness, and the rejection of the claims under U.S.C. § 103(a) should be withdrawn.

Evidence of Unexpected Result

In their specification, Applicants describe methods of administering an effective amount of a nucleic acid encoding at least one angiogenic protein or an effective fragment thereof into the myocardial tissue and administering to the mammal an effective amount of at least one

angiogenic factor or an effective fragment thereof to induce new blood vessel growth in an ischemic myocardial tissue of the mammal. Such methods provided a significant and unexpected improvement over the prior art. At page 5, lines 24-27, Applicants state:

> In particular, we have found that GM-CSF and other hematopoietic factors increase EPC mobilization and enhances neovascularization. **This observation was surprising and unexpected in light of prior reports addressing GM-CSF activity in vitro and in vivo.** Accordingly, this invention provides methods for using GM-CSF to promote EPC mobilization and to enhance neovascularization, especially in tissues in need of EPC mobilization and/or neovascularization. (Emphasis added.)

Applicants indicated that combination therapy of a vascularization modifying agent, such as GM-CSF, and an angiogenic protein is likely to provide a synergistic effect. Applicants state, "In many settings, it is believed that co-administration of the vascularization modulating agent and the angiogenic protein can positively impact neovascularization in the mammal, e.g., by providing additive or **synergistic effects.**" (page 8, lines 15-18; Emphasis added.) As further evidence that the combination is surprisingly more potent than expected, Applicants provide herewith Kawamoto et al., (Circulation 110:1398-1405, 2004, entitled "Synergistic Effect of Bone Marrow Mobilization and Vascular Endothelial Growth Factor-2 Gene Therapy in Myocardial Ischemia;" hereinafter "Kawamoto," Exhibit A).

Kawamoto describes the use of angiogenic gene therapy in combination with cytokine-induced endothelial progenitor cell mobilization in a swine model of chronic myocardial ischemia and a murine model of acute myocardial infarction. Kawamoto treated animals with chronic myocardial ischemia (swine) or with acute myocardial infarction with VEGF in combination with cytokines (e.g., GM-CSF and SCF), Kawamoto found that "In chronic myocardial ischemia, **combination therapy resulted in superior improvement in all indexes of perfusion and function** compared with all other treatment groups." (Kawamoto, p. 1398, Abstract under "Methods and Results"; emphasis added)

None of the prior art references cited by the Examiner teach or suggest that combination therapy would be so surprisingly effective for the treatment of **myocardial ischemia.** Where Applicants are able to show that the claimed invention is unexpectedly superior to the prior art the unexpected results are sufficient to show that the invention is nonobvious.

> One way for a patent applicant to rebut a prima facie case of obviousness is to make a showing of "unexpected results," i.e., to show that the claimed invention exhibits some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or unexpected. The basic principle behind this rule is straightforward—that which would have been surprising to a person of ordinary skill in a particular art would not have been obvious. *In Re Pravin L. Soni* 54 F.2d 746 at 750; 34 U.S.P.Q.2d 1684 (Fed. Circ. 1995; Emphasis added).

Furthermore, the mere presence of unexpected results is evidence that the claimed invention is nonobvious.

> Indeed, for many inventions that seem quite obvious, there is no absolute predictability of success until the invention is reduced to practice. There is always at least a possibility of **unexpected results, that would then provide an objective basis for showing that the invention, although apparently obvious, was in law nonobvious**. In re Patrick H. O'Farrell, 853 F.2d 894 at 903; 7 U.S.P.Q. 2d 1673 at (Fed Circ. 1988)

In sum, Applicants have provided further evidence showing that the results obtained with the claimed invention are superior to what was expected. Accordingly, the obviousness rejection of the claims should be withdrawn.

Double Patenting Rejection

Claims 49-61 and 63-66 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 49, 52-56, 58-65, and 68 of co-pending U.S. Application No. 10/696,391. Applicants submit that upon consideration and entry of the instant Amendment and Response, the provisional double patenting rejection will be the only rejection remaining in the instant application. Therefore, pursuant to M.P.E.P. §822.01, Applicants respectfully request that the provisional obviousness-type double patent application be withdrawn so that the instant application may proceed to allowance.

CONCLUSION

In view of the above amendment and Remarks, Applicants believe the pending application is in condition for allowance. If the Examiner disagrees, Applicants respectfully request that the Examiner contact the undersigned agent by telephone to schedule an interview prior to the mailing of an Office action.

Applicants believe that no fee is due to consider the present amendment. Nevertheless, the Director is hereby authorized to charge or credit any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 04-1105.

Dated: December 26, 2006

Respectfully submitted,

By _____

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Exhibit A

Synergistic Effect of Bone Marrow Mobilization and Vascular Endothelial Growth Factor-2 Gene Therapy in Myocardial Ischemia

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Background—We performed a series of investigations to test the hypothesis that combining angiogenic gene therapy and cytokine (CK)-induced endothelial progenitor cell mobilization would be superior to either strategy alone for treatment of chronic myocardial ischemia.

Methods and Results—A swine model of chronic myocardial ischemia and a murine model of acute myocardial infarction were used in this study. In both models, animals were randomly assigned to 1 of 4 treatment groups: Combo group, intramyocardial vascular endothelial growth factor (VEGF)-2 gene transfer plus subcutaneous injection of CKs; VEGF-2, VEGF-2 gene transfer plus saline subcutaneously injected; CK, empty vector transfer plus CKs; and control, empty vector plus subcutaneous saline. Acute myocardial infarction was also induced in wild-type mice 4 weeks after bone marrow transplantation from enhanced green fluorescent protein transgenic mice to permit observation of bone marrow–derived cells in the myocardium after acute myocardial infarction. In chronic myocardial ischemia, combination therapy resulted in superior improvement in all indexes of perfusion and function compared with all other treatment groups. In the bone marrow transplant mice, double immunofluorescent staining revealed that the combination of CK-induced mobilization and local VEGF-2 gene transfer resulted in a significant increase in the number of bone marrow–derived cells incorporating into the neovasculature, indicating that recruitment and/or retention of bone marrow–derived progenitors was enhanced by mobilization and that local VEGF-2 gene transfer can provide signals for recruitment or incorporation of circulating progenitor cells.

Conclusions—Mobilization of endothelial progenitor cells with cytokines potentiates VEGF-2 gene therapy for myocardial ischemia and enhances bone marrow cell incorporation into ischemic myocardium. (***Circulation***. 2004;110:1398-1405.)

Key Words: cytokines ■ endothelial cells ■ ischemia ■ stem cells ■ vascular endothelial growth factor

Catheter-based, intramyocardial vascular endothelial growth factor (VEGF) gene transfer has been shown to induce therapeutic angiogenesis in preclinical models of myocardial ischemia[1,2] and to increase exercise tolerance time and decrease the incidence of anginal episodes in pilot studies in patients with chronic myocardial ischemia.[3] These favorable effects of VEGF gene transfer were accompanied by objective evidence of improvement of myocardial perfusion in a recent placebo-controlled, double-blind pilot clinical trial.[4] The principal mechanism of these effects was initially thought to be the formation of new blood vessels by sprouting and migration of preexisting endothelial cells in ischemic tissue.[5–8] However, extensive preclinical data have suggested that a portion of the effect of angiogenic cytokines (CKs) involves the mobilization and recruitment of precursor cells, capable of differentiation into endothelial cells, from the bone marrow (BM).[9,10] Recent clinical data have revealed that VEGF gene transfer is also accompanied by the mobilization of BM-derived endothelial progenitor cells (EPCs).[11] Moreover, extensive preclinical and early clinical data have revealed that EPCs may exert a therapeutic effect on ischemic tissue when administered systemically or locally.[12–16] Together, these data suggest that administration of exogenous angiogenic growth factors may stimulate both angiogenesis and vasculogenesis for therapeutic neovascularization.

Granulocyte colony stimulating factor (G-CSF) and stem cell factor (SCF) are CKs that have been clinically applied for mobilization of BM-derived hematopoietic stem cells into the systemic circulation.[17–19] Administration of G-CSF and SCF

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has been reported to improve left ventricular (LV) function in mice with acute myocardial infarction (MI) through increased homing of mobilized, BM-derived EPCs and cardiomyogenic progenitor cells into ischemic myocardium,[20] providing direct evidence that mobilization of BM progenitors might represent a viable strategy for preserving the integrity and restoring function in ischemic tissue. In the present study, we performed experiments to test the hypothesis that BM mobilization can augment VEGF gene transfer-induced therapeutic neovascularization by enhancing the contribution of BM-derived precursor cells.

Methods

Experimental Animals

All animals were handled in accordance with the guidelines of the Animal Care and Use Committee at St Elizabeth's Medical Center (Boston, Mass).

Thirty-two male Yorkshire swine (Pine Acre Rabbitry Farm, Norton, Mass) weighing 20 to 25 kg were used to induce chronic myocardial ischemia. After left thoracotomy, an ameroid constrictor (Research Instruments SW) was placed around the proximal portion of the left circumflex coronary artery (LCx) as previously detailed.[21,22]

Thirty BM transplant (BMT) animal models were also prepared as previously described as a means of documenting the kinetics of BM-derived cells in the ischemic myocardium.[23] In brief, female C57BL/6 mice received BM mononuclear cells from transgenic mice constitutively overexpressing enhanced green fluorescent protein (eGFP mice, C57BL/6-TgN[ACTbEGFP]1Osb, Jackson Laboratory)[24] after sublethal irradiation. Four weeks after BMT, by which time the BM of the recipient mice was reconstituted, BMT mice were used for experiments.

Mice were anesthetized with 2,2,2-Tribromoethanol (200 μL/g body weight IP), orally intubated with a 22G IV catheter, and ventilated with a respirator (Harvard Apparatus). A left intercostal thoracotomy was performed, and the ribs were retracted with 5-0 polypropylene sutures to open the chest. After the pericardium was opened, the left anterior descending coronary artery (LAD) was ligated distal to the bifurcation between the LAD and diagonal branch with 8-0 polypropylene sutures through a dissecting microscope. After positive end-expiratory pressure was applied to fully inflate the lung, the chest was closed with 7-0 polypropylene sutures. The overall survival ratio after MI was 86% at 4 weeks.

Administration of Plasmid Human VEGF-2 Gene and CKs

In the swine study, NOGA nonfluoroscopic LV electromechanical mapping was performed to guide injections to foci of myocardial ischemia 5 weeks after constrictor placement. The NOGA system (Cordis) of catheter-based mapping and navigation and has been previously described in detail.[7,25,26] Ischemic myocardium was defined as a zone with unipolar voltage higher than an automatically determined cutoff and linear local shortening of <3%.[21] This definition was consistent in all examinations throughout this study. Immediately after the ischemic territory was identified by NOGA mapping, 800 μg plasmid human VEGF-2 gene (phVEGF-2) in 3 mL PBS or 800 μg empty vector in 3 mL PBS was injected into 6 sites within the ischemic myocardium (500 μL to each site) using the NOGA injection catheter (MyoSTAR, Cordis). The rhG-CSF (5 $\mu g \cdot kg^{-1} \cdot d^{-1}$) and rhSCF (20 $\mu g \cdot kg^{-1} \cdot d^{-1}$) or control saline was injected subcutaneously in swine with myocardial ischemia for 7 days starting immediately after the intramyocardial gene transfer. Swine were randomly assigned to 1 of 4 treatment groups: Combo group (n=8), catheter-based intramyocardial gene transfer of 800 μg of phVEGF-2 and subcutaneous rhG-CSF (5 $\mu g \cdot kg^{-1} \cdot d^{-1}$) and rhSCF (20 $\mu g \cdot kg^{-1} \cdot d^{-1}$) for 7 days immediately after the gene transfer; VEGF-2 group (n=8), phVEGF-2 gene transfer and saline injection; CK group (n=8), empty vector transfer and subcutaneous rhG-CSF and rhSCF injection; and control group (n=8), empty vector transfer and saline injection.

In the mouse study, 100 μg phVEGF-2 or empty vector as control (both dissolved in 100 μL saline) was administered intramyocardially with a 30G needle distal to the LAD occlusion site immediately after LAD ligation. The combination of CKs with recombinant human (rh) G-CSF (50 ng/g body weight) and rhSCF (200 ng/g) or control saline was administered (subcutaneously) daily for a week (days 0 to 6) after MI (rhG-CSF and rhSCF were supplied by Amgen, Inc). The mice were randomly assigned to 4 subgroups (n=6 or 7 in each group): empty vector plus saline (control group), empty vector plus CKs (CK group), phVEGF-2 plus saline (VEGF-2 group), or phVEGF-2 plus CKs (Combo group).

Physiological Assessment of LV Function and Ischemia

In the swine study, transthoracic echocardiography (SONOS 5500), selective left coronary angiography, and NOGA LV electromechanical mapping were performed 5 weeks after constrictor placement (just before injection of genes) and 4 weeks after gene injection. Echocardiographic fractional shortening (FS) and regional wall motion scores[27] were quantified by use of the LV short-axis view at the midpapillary muscle level. Collateral flow to the LCx territory was graded angiographically in a blinded fashion with the Rentrop scoring system.[28] The area of ischemia was quantified by NOGA mapping as previously described.[3]

All data were evaluated by blinded observers (echocardiography by K.K., coronary angiography by S.S., and postprocessing analysis of the NOGA mapping by I.J.).

Histological Assessment of Neovascularization and LV Remodeling

All swine were killed 4 weeks after gene transfer. At necropsy, swine hearts were sliced in a bread-loaf fashion into 4 transverse sections from apex to base, and each section was separated to anterior, lateral, posterior LV free wall, interventricular septum, and right ventricular free wall. All tissues obtained from each segment were fixed in 100% methanol. Immunohistochemistry for isolectin B4 was also performed to evaluate capillary density in the ischemic myocardium identified by NOGA mapping.

Double Immunofluorescence Histochemistry

The hearts of GFP-BM transplanted mice were harvested at predetermined times after surgery and prepared for frozen tissue sections. Frozen cross sections (6-μm thickness) were air dried and fixed with 4% paraformaldehyde for 5 minutes. After washing with PBS, double immunohistochemistry was performed with antibodies against GFP and isolectin B4. Nonspecific protein binding was blocked with 10% normal goat serum. Rabbit polyclonal anti-GFP antibody (1:200 dilution; Molecular Probe) was used at 4°C overnight, followed by goat anti-rabbit IgG conjugated with Cy2 (1:500 dilution; Jackson ImmunoResearch) as a secondary antibody for 30 minutes at room temperature. The endothelial cell-specific marker, biotinylated isolectin-B4 (1:100 dilution; Vector Laboratories), was used as a second primary antibody for capillary staining and visualized by binding with rhodamine-conjugated streptoavidin (1:500 dilution; Jackson ImmunoResearch) for 30 minutes at room temperature. Normal rabbit IgG served as a negative control for GFP detection. Nuclei were counterstained with DAPI (1:5000) and mounted in aqueous mounting medium. Images were examined with a fluorescent microscope (Nikon ECLIPSE TE300).

Double-positive cells were quantified in 5 randomly selected fields from 5 sections from each heart. All morphometric studies were performed by 3 examiners (H.M., A.H., and M.I.) who were blinded to treatment assignment.

Statistical Analysis

All values are expressed as mean±SE. Student's paired *t* test was used to compare data before and after treatment. ANOVA was

performed to compare data among the 4 treatment groups. A value of $P<0.05$ was considered statistically significant.

Results

Two pigs died in the control and CK groups before the final assessment. There were no deaths in the VEGF-2 or Combo groups.

BM Mobilization Augments the Effects of VEGF-2 Gene Transfer for Attenuation of Chronic Myocardial Ischemia

In the swine study, the ischemic area determined by NOGA mapping before gene transfer was not significantly different between the Combo, VEGF-2, CK, and control groups (35.4±4.4%, 34.7±5.6%, 43.0±8.7%, and 26.5±6.2%, respectively). Four weeks after treatment, the reduction in ischemic area was significantly better in the Combo therapy group (−85.4±2.2%) compared with all other treatment groups (VEGF-2, −24.3±21.5%; CK, 29.5±48.3%; and control, 40.7±25.1%; $P<0.05$ versus VEGF-2, $P<0.01$ versus CK and control). Ischemic area was also significantly improved in the VEGF-2 group compared with the control group ($P<0.05$). The ischemic area in the CK group was not significantly different from that in the control group (Figure 1).

Cytokine-Induced BM Mobilization Enhances Therapeutic Neovascularization by VEGF-2 Gene Transfer

In the swine study, selective left coronary angiography was performed to evaluate collateral development before and after treatment. The mean Rentrop score of collateral development to the LCx territory at baseline was 1.3±0.3 in the Combo group, 1.4±0.3 in the VEGF-2 group, 2.0±0.4 in the CK group, and 1.0±0.3 in the control group (P=NS). The change in the Rentrop score after treatment was significantly greater in the Combo group than in the control group ($P=0.01$). The change in the Rentrop score in the Combo group was similar to those in VEGF-2 and CK groups; however, neither the VEGF-2 nor the CK group showed an improvement in Rentrop scores that was significantly greater than the controls (Figure 2). These data indicated that there was anatomic evidence of improved collateral formation in the Combo therapy group compared with all other treatment groups.

In the swine study, histochemical staining for isolectin B4 was performed to identify capillaries in the ischemic myocardium 4 weeks after treatment. Capillary density was significantly greater in the Combo group than in the VEGF-2, CK, and control groups (879.9±44.8, 717.0±75.7, 326.4±14.1, and 345.0±20.4/mm^2, respectively; $P=0.03$ versus VEGF-2, $P<0.0001$ versus CK and control). Capillary density was also significantly greater in the VEGF-2 group than in the CK and control groups ($P<0.0001$). Capillary density in the CK group was similar to that in the control group (Figure 3). These data reveal that in addition to augmenting the angiographically visible collateral supply, combined VEGF-2 myocardial gene therapy plus CK-induced mobilization of BM progenitors resulted in a significant increase in microvascular capillary density compared with monotherapy.

BM Mobilization Augments the Effects of VEGF-2 Gene Transfer on LV Function in Chronic Myocardial Ischemia

In the swine study, echocardiographic FS and regional wall motion score before treatment were similar in all groups (FS: Combo, 27.6±1.3%; VEGF-2, 29.6±0.9%; CK, 30.5±1.4%; control, 29.4±1.2%; regional wall motion score: Combo, 22.4±1.0; VEGF-2, 20.8±0.7; CK, 20.4±0.5; control, 20.5±0.8). The improvement in FS after treatment was significantly greater in the Combo group than in the VEGF-2, CK, and control groups (5.3±0.9%, 1.0±1.2%, −1.1±0.8%, and −1.1±1.3%, respectively; $P=0.03$ versus VEGF-2, $P=0.01$ versus CK, $P=0.001$ versus control). Changes in FS were similar in the VEGF-2, CK, and control groups. Regional wall motion score after treatment was significantly improved in the Combo group compared with the VEGF-2, CK, and control groups (−3.9±1.0, −1.3±0.9, 0.4±0.5, and 1.2±0.9, respectively; $P=0.04$ versus VEGF-2, $P=0.009$ versus CK, and $P=0.0004$ versus control; Figure 4a and 4b).

Cytokine Mobilization Increases Recruitment and Incorporation of BM Cells Into Myocardial Neovasculature

Immunohistochemistry was performed on the hearts from BMT mice 1 week after MI to assess BM-derived cell incorporation into the neovasculature. Double immunofluorescent staining for eGFP and Isolectin B4 permitted identification of BM-derived cells that also expressed a marker of endothelial cell identity (Figure 5A). The double-positive cells were quantified and were found to be most abundant in the border zones between ischemic and nonischemic tissue in the Combo group (50.7±5.8), followed by the VEGF-2 group (19.8±3.7). Both groups had significantly greater numbers of double-positive cells than the control group ($P<0.001$), and the number of double-positive cells in the Combo group was significantly greater than in the VEGF group ($P<0.01$; Figure 5B). As shown in Figure 5A, some of the double-positive cells were incorporated into tubular structures, consistent with vasculogenesis. These data provide evidence that VEGF gene therapy stimulates vasculogenesis in the myocardium and that this effect can be augmented by BM mobilization.

Discussion

The concept of therapeutic angiogenesis by administration of angiogenic genes or proteins has been established in numerous preclinical models.[1,5,8] Recently, pilot clinical trials of therapeutic angiogenesis using some of these growth factors have been reported in patients with coronary artery disease.[4,29–31] Although subjective symptoms have been significantly improved in these phase I and II trials, some studies have failed to demonstrate significant improvement in objective findings such as myocardial perfusion and exercise tolerance. Analysis of the data generated in all these pilot studies reveals at least 2 common features: (1) In each study, the effect of a single agent was evaluated, and (2) certain patients are "nonresponders." The absence of a response in certain individuals is a consistent feature of all therapies and is the basis for the concept of pharmacogenomics, the science of designing drugs based on genetic features of individual

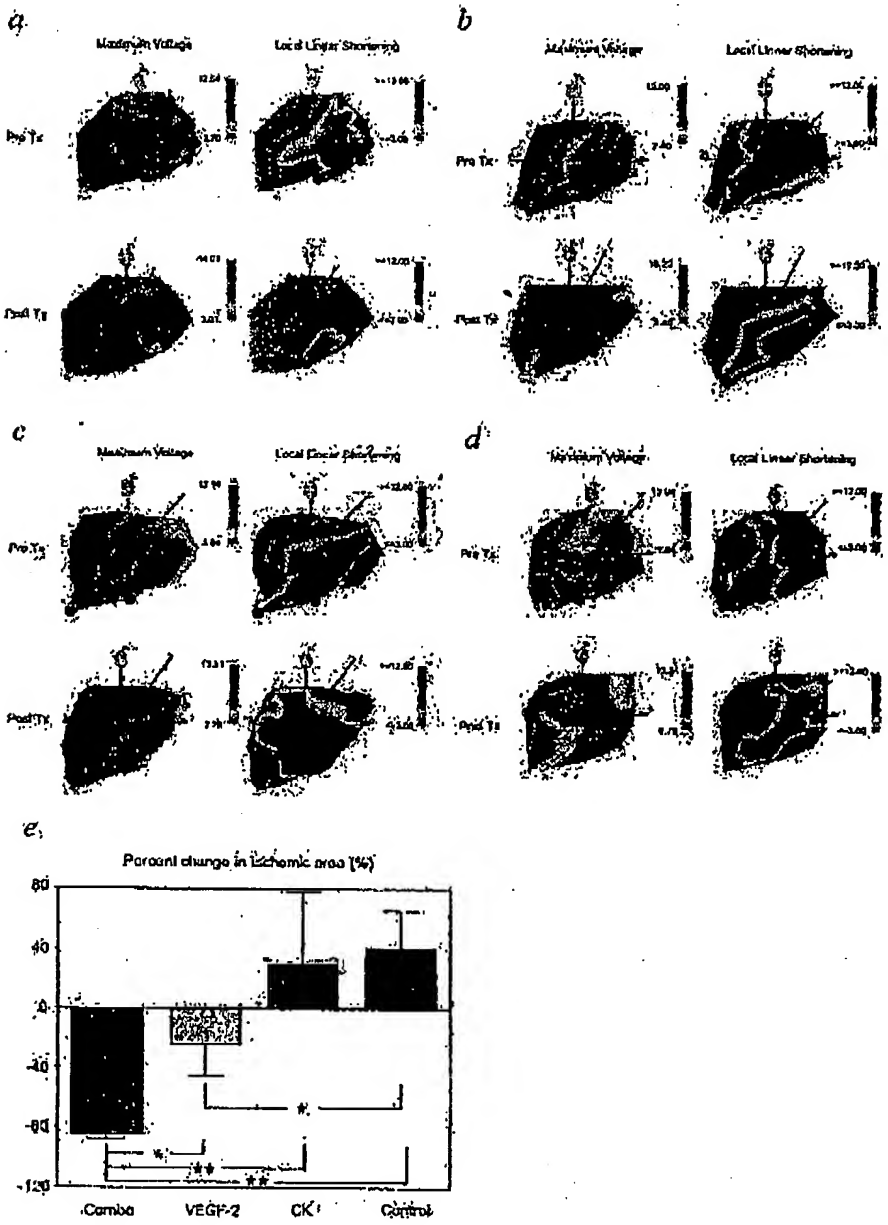

Figure 1. Representative recordings of NOGA electromechanical mapping immediately before (pre TX) and 4 weeks after (post Tx) gene transfer in porcine model of chronic myocardial ischemia. Black dots in pretreatment map show sites of gene transfer. Red area on pretreatment linear local shortening map (top right) indicates area of decreased wall motion in lateral wall of left ventricle, consistent with ischemia in territory of LCx. Four weeks after gene transfer, this area of ischemia improved in representative case in Combo therapy group (a) and moderately in case from VEGF-2 group (b), whereas no improvement was observed in cases from CK group (c) and control group (d). e, Percent change in ischemic area during 4 weeks after gene transfer. $^{*}P<0.05$; $^{**}P<0.01$.

patients. Lacking this tailored approach to drug development, physicians have traditionally tried combining drugs to achieve therapeutic effects in patients with conditions refractory to single agents.

In parallel with studies attempting neovascularization by administration of angiogenic CKs, the use of progenitor or stem cells as therapeutic agents in ischemic diseases has emerged.[13,14,32] These studies are based on observations indicating that circulating cells, some of which appear to originate in the BM, are capable of homing to and augmenting neovascularization of ischemic tissue.[33-35] More recent data have indicated that at least part of the effect of locally administered angiogenic CKs results from recruitment of progenitor cells and that the failure of native or therapeutic

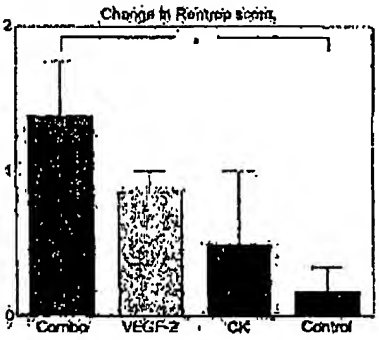

Figure 2. Change in Rentrop grade of collateral development 4 weeks after gene transfer in porcine model of chronic myocardial ischemia. *$P<0.05$.

neovascularization might result in part from a deficiency in the quantity or quality of these cells.[11,36–38]

This constellation of findings raised an important fundamental question regarding VEGF gene therapy for therapeutic neovascularization: Is the mechanism of local VEGF predominantly via local effects, enhancing the proliferation and migration of EC in pre-existing blood vessels, or is it possible that VEGF, expressed after gene transfer in the local tissue environment, is acting as a chemokine, recruiting progenitor cells from remote sites to deliver a more varied repertoire of CKs[39] in addition to providing parent cells for the neovasculature?[33] The latter possibility is well illustrated in studies by Orlic et al[40] in the setting of acute ischemia in which the local homing signals for circulating cells are apparently robust, obviating the need for induction of local CK expression.

Accordingly, we hypothesized that the effect of transient local expression of VEGF, mediated by gene transfer of naked plasmid DNA, might be amplified by increasing the circulating supply of progenitor cells by systemically administered hematopoietic stem cell mobilization using GCSF and SCF. This is consistent with a report demonstrating superiority of a combination of growth factor therapy and cell transplantation. In this previous study,[41] the combination of hepatocyte growth factor gene transfer and neonatal rat cardiomyocyte transplantation had more potent therapeutic efficacy in a model of rat MI compared with either single treatment.

Although the therapeutic potential of systemically administered, mobilizing CKs has been reported in the setting of acute MI,[20] the efficacy of the same approach in chronic myocardial ischemia has not been defined in animal models. Interestingly, this approach has been attempted in a single human pilot study of granulocyte-macrophage CSF administration.[42] This study revealed potential benefit by a novel

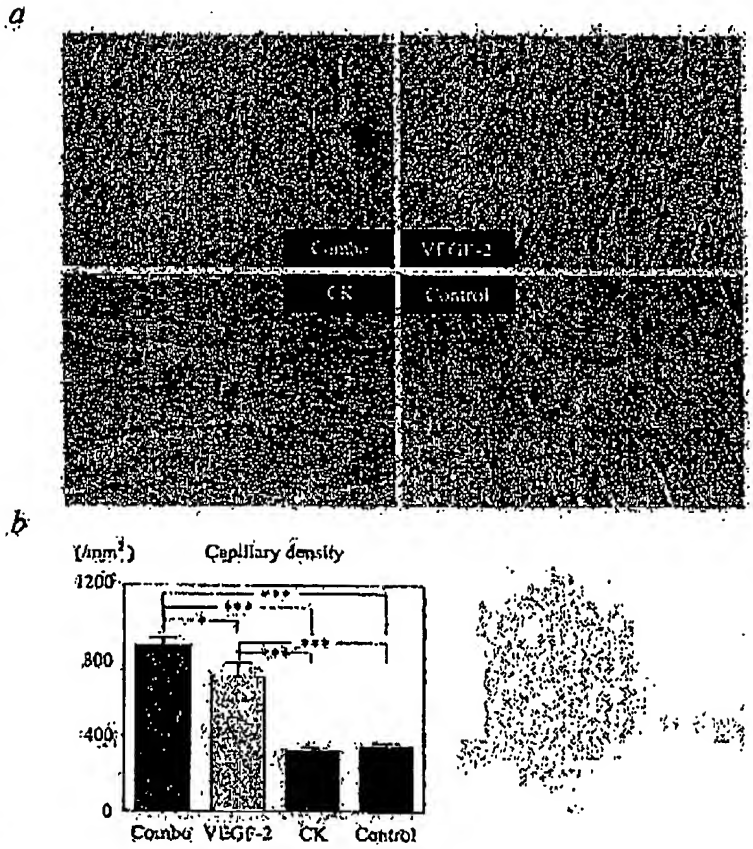

Figure 3. a, Representative immunohistochemistry for isolectin B4 in specimens of ischemic porcine myocardium from 4 treatment groups. These specimens were obtained 4 weeks after gene transfer. b, Capillary density 4 weeks after gene transfer. *$P<0.05$; ***$P<0.001$.

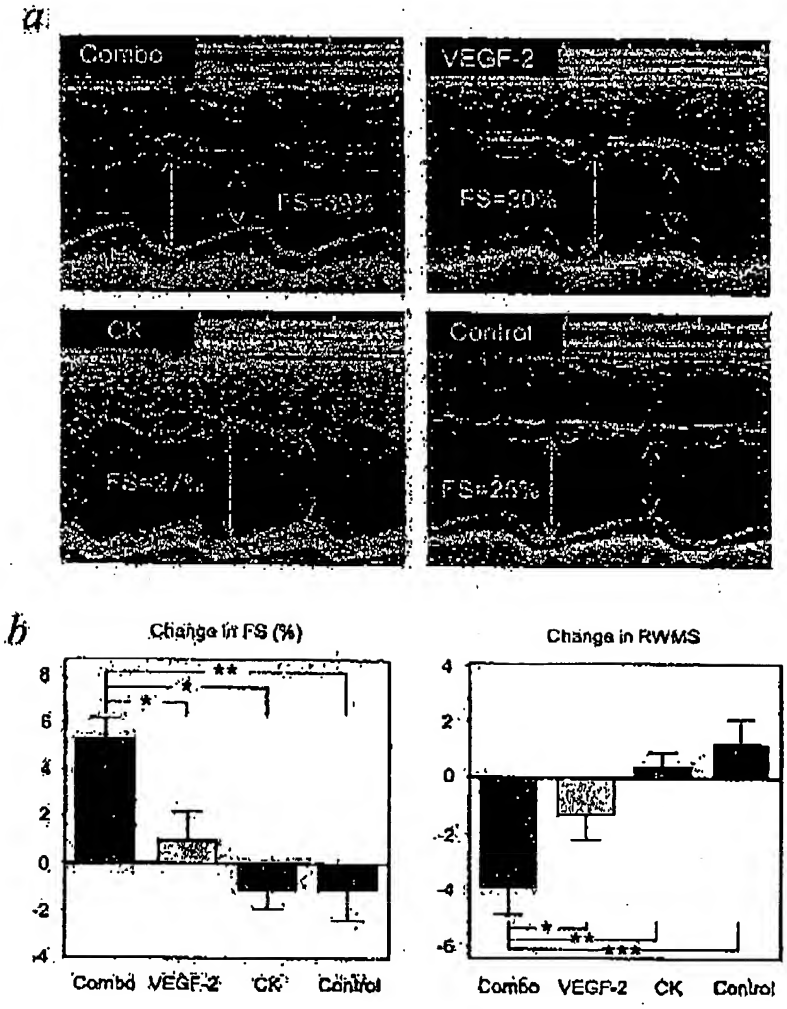

Figure 4. a, Representative findings of M-mode echocardiography 4 weeks after gene transfer in porcine chronic myocardial ischemia. b, Changes in FS and regional wall motion score during 4 weeks after the gene therapy in swine in Combo, VEGF-2, CK, and control groups. RWMS indicate regional wall motion score. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

method of coronary flow measurement, but no change in symptoms or physiologically induced ischemia was reported, and these initial findings have not yet been repeated or extended in further studies.

In our swine study, monotherapy with CKs failed to attenuate chronic myocardial ischemia, to increase vascularity in the ischemic myocardium, or to improve LV function. In contrast, as documented previously, monotherapy with VEGF-2 gene transfer significantly improved chronic myocardial ischemia as documented by NOGA mapping, improved capillary density, and resulted in a favorable trend in LV functional improvement. The results of VEGF-2 gene transfer were consistent with previous reports in preclinical and pilot clinical trials.[3,4] Most notably, however, the combination of VEGF-2 gene transfer plus CKs was superior to the monotherapies in terms of neovascularization and LV functional recovery. These favorable outcomes support the notion that progenitor cells play a key role in VEGF-induced local tissue revascularization and that the combination of BM mobilization and gene therapy can achieve superior therapeutic neovascularization.

To provide additional evidence for the enhanced contribution of BM-derived cells after combination therapy, BMT from cGFP mice into wild-type mice was performed. Histological examination revealed greater numbers of BM-derived cells in the myocardial neovasculature in mice receiving combination therapy than in those receiving monotherapy. These findings are consistent with prior observations. VEGF-1 has previously been shown to enhance mobilization of BM-derived EPCs into the circulation and to increase the incorporation of EPCs into sites of neovascularization.[22] Intramyocardial VEGF-2 gene transfer also increased circulating EPC counts.[11] These and other prior studies suggested that progenitor cells were an integral component of ischemia- and CK-induced neovascularization of ischemic tissues. The present findings provide additional evidence to support a fundamental role for EPCs in ischemia-induced neovascularization and suggest that therapies directed at enhancing the supply of these cells may be helpful in addressing the failure of native or CK-induced collateral vessel formation. Moreover, the failure of CK-induced EPC mobilization as a monotherapy in the setting of chronic ischemia indicates that

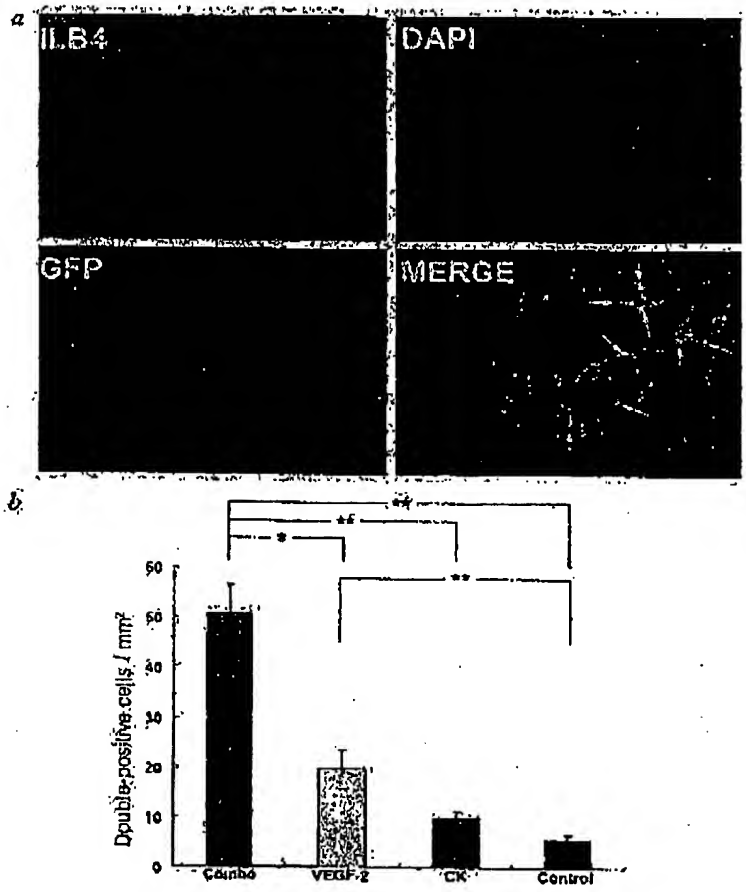

Figure 5. a, Representative double immunofluorescent microscopic findings from BM transplanted mice 1 week after MI. This example shows myocardial tissue from mouse in Combo group stained with antibody to isolectin B4 (ILB4) to identify endothelial cells, DAPI to identify nuclei, and antibody to GFP to identify BM-derived cells. In merge image, arrows identify yellow, resulting from overlap of red (ILB4 positive, endothelial cells) and green (GFP, BM-derived cells), thus identifying BM-derived cells that have relocated to myocardium and now express endothelial marker. b, Quantification of double-positive cells, revealing that number of double-positive cells in Combo group was greater than all other treatment groups and that VEGF group also had greater number of double-positive cells than controls. *$P<0.01$; **$P<0.001$.

a local signal, in this case provided by VEGF gene therapy, is required for recruitment and incorporation of circulating progenitors. The precise mechanisms governing the recruitment, retention, and incorporation of BM-derived progenitors into the myocardial tissue and the relative roles of each in the enhanced functional recovery documented remain to be elucidated.

Together, these findings underscore the likelihood that progenitor cells must be considered not only as a part of the native mechanisms that govern vascular biology but also as entities whose failure may play a fundamental role in the advent of vascular pathology.[38] Modulation of progenitor cell function therefore represents a reasonable therapeutic target for treatment of ischemic diseases.

Acknowledgments

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